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Inhibition of Corticotropin Releasing Hormone Type-1 Receptor Translation by an Upstream AUG Triplet in the 5′ Untranslated Region

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ABSTRACT

The influence of an upstream open reading frame (ORF) in the 5′-untranslated region (UTR) of the mRNA on corticotropin-releasing hormone receptor type 1 (CRHR1) translation was studied in constructs containing the 5′-UTR of CRHR1, with or without an ATG-to-ATA mutation in the upstream ORF, and the main ORF of luciferase or CRHR1. Upstream mutation in luciferase constructs increased luciferase activity when transfected into COS-7 or AtT20 cells compared with the native 5′-UTR. Transfection of CRHR1 constructs containing the upstream mutation into AtT20 or LVIP2.0zc reporter cells, resulted in higher ^{125}I-Tyr-oCRH binding and corticotropin-releasing hormone-stimulated cAMP production, without changes in CRHR1 mRNA levels (measured by RNase protection assay). In vitro translation of luciferase or CRHR constructs with or without mutation of the upstream ATG, and Western blot analysis with anti-luciferase and anti-CRHR1 antibodies confirmed that mutation of the upstream ATG increases translation of the main ORF. The mechanism by which the upstream ORF inhibits translation may involve translation of the upstream peptide, because in vitro translation, or transfection into LVIP2.0zc cells of a fusion construct of the upstream ORF and green fluorescent protein (GFP) yielded a band consistent with the molecular size of GFP protein. The study shows that the upstream AUG in 5′-UTR of CRHR1 mRNA inhibits receptor expression by inhibiting mRNA translation and suggests the short open reading frame in the 5′-UTR plays a role in regulating translation of the CRH receptor.

The 41-amino-acid hypothalamic peptide corticotropin-releasing hormone (CRH), produced by parvocellular neurons of the hypothalamic paraventricular nucleus is a major regulator of ACTH secretion during activation of the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1983; Antoni, 1986). CRH initiates its biological effects by binding to specific, high-affinity plasma membrane receptors located in the pituitary corticotroph and other target tissues (DeSouza and Kuhar, 1986; Aguilera et al., 1987). Two major CRH receptor subtypes have been identified by molecular cloning: type 1 (CRHR1), which is widely distributed in the brain and is the only subtype in the pituitary corticotroph, and type 2 (CRHR2), which is present in discrete areas of the brain and in the periphery (Lovenberg et al., 1995; Perrin et al., 1995). Both CRH receptor subtypes belong to the 7 transmembrane domain, G protein receptor family coupled to adenylate cyclase (Aguilera, 1994).

CRH receptors in the pituitary undergo changes during manipulation of the HPA axis, but it should be noted that binding down-regulation occurs regardless of whether there is stimulation (chronic stress, adrenalectomy) or inhibition (glucocorticoid administration) of the HPA axis (Aguilera, 1994). Moreover, analysis of the changes in CRHR1 mRNA has shown no correlation between steady-state mRNA levels and receptor content in the pituitary (Aguilera, 1998). For example, CRH receptor loss in all the above conditions is associated with normal or elevated CRHR1 mRNA levels (Rabadan-Diehl et al., 1996, 1997). This indicates that CRH receptor levels are mainly regulated at the translational and post-translational levels and that mRNA levels are not the determining factor.

It has been shown that the presence of short open reading frames (ORF) in the 5′-UTR of the mRNA can affect translation of the main ORF in a number of transcripts (Kozak,

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ABBREVIATIONS: CRH, corticotropin-releasing hormone; HPA, hypothalamic-pituitary-adrenal; UTR, untranslated region; ORF, open reading frame; GFP, green fluorescent protein; uORF, upstream open reading frame; CRHR, corticotropin-releasing hormone receptor; bp, base pair(s); PCR, polymerase chain reaction; DTT, dithiothreitol.

1991; Geballe and Morris, 1994). Although this occurs in less than 10% of transcripts in vertebrates, it is more frequent in low abundance proteins, such as some members of the G protein-coupled receptor family, including muscarinic, adrenergic, serotonergic, substance P, substance K, and angiotensin II receptors (Curnow et al., 1995; Kobilka et al., 1987; Kozak, 1991). For β_2-adrenergic and type 1 angiotensin II receptors, it has been reported that AUG triplets that are part of upstream open reading frames (uORFs) in the 5′-UTR of the transcript inhibit translation of the receptor protein (Parola and Kobilka, 1994; Mori et al., 1996). The 5′-UTR of the CRHR1 mRNA contains about 220 bp, and sequence analysis reveals a short ORF located between bp −50 and −16 upstream of the main ORF initiating methionine, potentially encoding a 10-amino-acid peptide. The structure of the 5′-UTR of the CRHR1, and especially the 33-bp uORF, is highly conserved between species, including human, rat, and mouse, suggesting that it is an important regulatory feature of the mRNA (Chen et al., 1993; Perrin et al., 1993; Vita et al., 1993). Because uORFs in the 5′-UTR can influence translation of the main ORF, it is possible that the uORF of the CRHR1 inhibits translation of CRHR1 mRNA and contributes to CRH receptor down-regulation in the presence of high CRHR1 mRNA levels. In this study, we investigated whether mutational inactivation of the upstream AUG of the CRHR1 5′-UTR affects translation of CRHR1 and examined possible mechanisms by which the uORF can influence this event.

Materials and Methods

CRHR1 5′-UTR Mutants and Fusion Protein Constructs. A 2.5-kilobase full cDNA clone of the rat CRHR1, kindly provided by Dr. W. Vale (Salk Institute, La Jolla, CA) was used as template for PCR amplification of DNA fragments used for all constructs. First, a fragment corresponding to the 5′-UTR of the CRHR1 was generated by PCR using primers F1 (5′- GCA AGC TTG AGT CGG GAG AAG GCT ACC AG -3′) and R1 (5′- CTG GAT CCG TCA GCG TCC TGG ATG CC -3′), and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The resulting construct was named p5UTR. A second construct, p5UTRm, was generated by a similar approach, but the ATG codon of the upstream open reading frame (uORF), located 50 bp upstream from the initiating methionine, was mutated into an ATA using primers F1 and R2 (5′- TGG ATC CGT CAG CGT CCT GGA TGC CTG GAT CGC TCC GAT ATC CCA GAG −3′). The constructs p5UTR and p5UTRm were used to generate constructs with the coding regions of the luciferase reporter gene or CRHR1 for expression and transcription studies (Fig. 1).

Constructs p5UTR-CRHR1 and p5UTRm-CRHR1 were created by PCR amplification of the coding region of the CRHR1 and 3′UTR using primers F2 (5′-CGG ATC CAG CCC GAC CAT GGG ACG GCG CCC GCA GC-3′) and SP6 (5′-GCA TTT AGG TGA CAC TAT AGT-3′), and subcloning into p5UTR and p5UTRm, respectively. Luciferase constructs were created by PCR amplification of the coding region of luciferase with the upstream primer, 5′- ACG GAT CCA GCC CGA CCA TGG AAG ACG CCA AAA -3′, and the downstream primer, 5′- GAC GAT AGT CAT GCC CCG CG -3′, and the luciferase pGL3-Basic vector (Promega, Madison, WI) as template. The luciferase PCR fragment was subcloned into p5UTR and p5UTRm to generate p5UTR-luc and p5UTRm-luc, respectively.

Fusion constructs of the ORFs of the upstream peptide and green fluorescent protein (GFP), p5UTRgfp-F, and the ATG mutated upstream ORF and GFP, p5UTRmgfp-F (negative control) were created as follows. First, the initiating methionine codon for the GFP gene, ATG, was mutated into TCC by amplifying the GFP coding region from vector pEGFP-N3 (CLONTECH, Palo Alto, CA) using a pair of primers containing the ATG-to-TCC mutation (upstream primer, 5′-CCG GAT CCG TGA GCA AGG GCG AGG CGC T -3′, downstream primer, 5′-ATG ATC TAG AGT CGC GGC CGC TTT -3′). The amplified product was subcloned between the *Bam*HI and *Xba*I sites of pcDNA3.1(+), to generate a construct named pGFPm. Secondly, the stop codons (TGA) in the uORF of p5UTR and p5UTRm were mutated into GGA by PCR using primers F1 and R3 (5′-CTG GAT CCG CGT CCT GGA TGC CTG GAT CG -3′). The PCR products were subcloned between *Hin*dIII and *Bam*HI sites of pGFPm.

The accuracy of all constructs shown in Fig. 1 was confirmed by nucleotide sequencing using a dideoxy DNA sequence analysis (DNA sequenase Kit; PE Applied Biosystems, Foster City, CA).

Cell Culture and Transfections. COS-7 and AtT-20 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 mg/l streptomycin, at 37°C under 95% air/5% CO_2. LVIP2.0zc cells were cultured in the same medium containing an additional 25 mg/l hygromycin B. Cells were grown to 80% confluence and transfected using Lipofectamine PLUS reagent (Life Technologies, Gaithersburg, MD). All cell culture reagents were also purchased from Life Technologies (Gaithersburg, MD).

Luciferase Activity Assay. COS-7 and AtT-20 cells cultured in 24-well plates to 80% confluence were transfected with 0.4 μg per well of p5UTR-luc or p5UTRm-luc plasmid DNA. Cotransfection with the renilla luciferase vector, pRLsv40, (Promega) at a 1:50 ratio was performed to correct for transfection efficiency. Twenty four hours after transfection, cells were harvested for measurement of firefly and renilla luciferase activities using a dual-luciferase assay system (Promega) in a Monolight 20101 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Measurement of Functional CRHR1. The content of biologically active CRHR1 was quantified by binding of ^{125}I-Tyr-oCRH

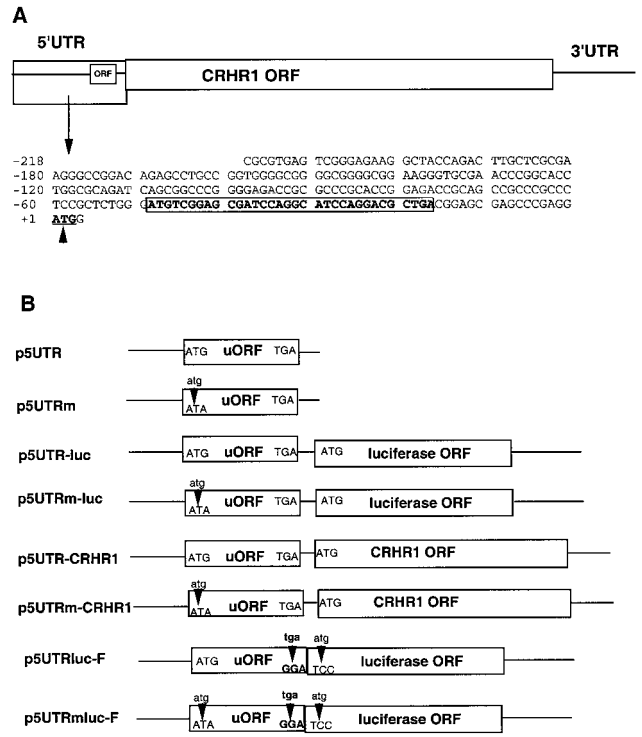

Fig. 1. Schematic depiction of the CRHR1 cDNA (A) showing boxes corresponding to the uORF in the 5′-UTR, the main ORF encoding the CRHR1 (CRHR1 ORF). The sequence of the 5′-UTR below highlights the uORF and the initiation codon of the main ORF. The diagrams in B show the structure of the different wild-type and mutant (m) constructs used in the experiments described under *Materials and Methods* and *Results*. The mutations are shown by the arrows, and the two fusion constructs at the bottom are indicated by -F at the end.

(Wynn et al., 1983), and by the ability of CRH to stimulate β-galactosidase activity in LV1P2.0zc cells (Koenig et al., 1991) transiently transfected with CRHR1. For the binding assay, LVIP2.0zc cells (kindly provided by Dr. Monica Koenig, National Institute of Mental Health, Bethesda, MD), plated in 72-cm^2 flasks, were transfected using 10 μg of p5UTR-CRHR1 or p5UTRm-CRHR1 per flask. Forty-eight hours after transfection, cells were harvested and homogenized in 50 mM Tris·HCl, pH 7.4, containing 100 kIU/ml aprotinin and 1 mM DTT by 6 strokes in a Teckmar mechanical homogenizer (Teckmar, Cincinnati, OH). After centrifugation for 10 min at 1,000*g*, supernatants were centrifuged for 30 min at 30,000*g*, and membrane pellets resuspended in the same buffer to a concentration of 0.5 to 1.0 mg of protein/ml. Aliquots of 100 μl of membrane suspension were incubated with 100,000 cpm of ^{125}I-Tyr-oCRH (NEN, Boston, MA) and increasing concentrations of unlabeled oCRH (0 to 100 nM), at room temperature for 1 h in a total volume of 300 μl of 50 mM Tris·HCl, pH 7.4, containing 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 100 kIU/ml aprotinin, and 0.1% BSA (Wynn et al., 1983), in 1.5-ml Microfuge tubes. Bound radioactivity was separated by centrifugation at 14,000*g* for 3 min after addition of ice-cold PBS containing 7.5% polyethylene glycol. The pellet was washed twice by addition of 1 ml of the same buffer followed by centrifugation for 1 min at 14,000*g*, and the tip of the tubes severed and counted in a γ-spectrometer. Receptor number and binding affinity were calculated by computer analysis of the binding inhibition curves using the computer program LIGAND (Munson and Rodbard 1980).

The expression of functional receptors was further studied using a colorimetric assay that uses cAMP-dependent transcription of a β-galactosidase reporter gene to quantify the levels of intracellular cAMP in LVIP2.0zc cells after stimulation with CRH. This cell line, stably transfected with the β-galactosidase reporter gene under the control of a promoter containing cAMP responsive elements, has been used to measure cAMP production after transfection of the cells with adenyl cyclase-coupled receptors (Koenig et al., 1991; Liaw et al., 1997). LVIP2.0zc cells grown in 24-well plates at 80% confluence were transfected with either p5UTR-CRHR1 or p5UTRm-CRHR1. Cells were changed to serum-free medium containing 0.1% BSA 18 h after transfection, exposed to increasing concentrations of oCRH for 6 h, and washed twice with PBS before harvesting for measurement of β-galactosidase activity using the β-galactosidase Enzyme Assay System (Promega) and spectrometry at 420 nm. EC_{50} values were calculated using GraphPad Prism (GraphPad Software, San Diego, CA).

RNase Protection Assay. To determine whether the upstream ATG mutation affects transcriptional activity, the amounts of mRNA produced after transfection with the different constructs was measured by RNase protection assay. LVIP2.0zc cells grown in 72-cm^2 flasks were transfected either with p5UTR-CRHR1, p5UTRm-CRHR1 or pcDNA3.1(+) as a negative control. Total RNA was isolated using TRIzol Reagent (Life Technologies) 48 h after transfection. Hybridization was performed using reagents from the RNase Protection Assay Kit (Roche Diagnostics, Mannheim, Germany). Ten micrograms of total RNA were hybridized with 300,000 cpm of a ^{32}P labeled antisense CRHR1 riboprobe corresponding to 602 bp of the rat CRHR1 coding region (Luo et al., 1994), and 3,000 cpm of human 18 S ribosomal RNA riboprobe to correct for RNA loading in the gel, in a total volume of 30 μl. After hybridization for 4 h at 45°C, the reaction mixture was subjected to RNase treatment by addition of 3.5 μg of RNase A and 50 U of RNase T1 (RNase protection kit; Roche Diagnostics), and the RNA:RNA hybrids were electrophoresed in a 6% polyacrylamide/7 M urea gel. The gel was dried and exposed overnight to a FUJI imaging plate type MacBAS V2.52 (Fuji Photo Film Co. Ltd, Tokyo, Japan) to quantify the intensity of the protected bands using a Fuji Bioimaging analyzer. Data are expressed as arbitrary Fuji units, which are directly proportional to the radioactivity present in the bands, normalized by 100 18S Fuji units (Miyahara, 1989).

RNA Single-Stranded Conformational Polymorphism. To determine whether modifications in the ATG of the uORF in the 5′-UTR of the CRH receptor influences mRNA structure, p5UTR and p5UTRm constructs were transcribed using [^{32}P]UTP in an in vitro transcription system (Promega). The transcription reaction was performed as described by Sarkar et al. (1992), in a total volume of 20 μl of 40 mM Tris·HCl, pH 7.9, containing 10 mM NaCl; 6 mM $MgCl_2$; 10 mM DTT; 2 mM spermidine; 0.05% Tween 20; 20 U RNasin; 0.5 mM each ATP, GTP, and CTP; 12 uM UTP; 0.5 μg of linearized template, 50 μCi of [^{32}P]UTP and 20 U of T7 RNA polymerase. After 1 h incubation at 37°C, the reaction was terminated by placing the tube on ice. One microliter of the ^{32}P-labeled RNA was mixed with 4 μl of 50% formamide, 2 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue. The mixture was heated at 70°C for 5 min, cooled on ice for 5 min, and 1 μl was electrophoresed through a 5.6% nondenaturing polyacrylamide gel at 4°C with precooled 1× Tris/borate/EDTA buffer. After 4 to 6 h of electrophoresis at 1200 V, the gel was dried and exposed to Kodak X-Omat AR X-ray film (Eastman Kodak, Rochester, NY) for 2 or 5 h.

Western Blot Analysis of CRHR1, Luciferase, and GFP. The relative amounts of CRHR1 protein produced after transfection of the cells with p5UTR-CRHR1 or p5UTRm-CRHR1 were measured by immunoblot. LVIP2.0zc cells were cultured in 75-cm^2 flasks, transfected with 10 μg of either p5UTR-CRHR1 or p5UTRm-CRHR1 and 48 h later harvested and pelleted at 1,000*g* for 5 min. Membrane-rich fractions were prepared by homogenization in 1 ml of 50 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 1 μg/ml aprotinin, by 12 strokes in a Dounce glass/glass homogenizer. After centrifugation for 5 min at 800*g* to discard nuclei and unbroken cells, the supernatant was centrifuged at 30,000*g* for 30 min. Membrane pellets were resuspended in homogenization buffer containing 1% Triton X-100, gently shaken for 90 min at 4°C, and centrifuged at 14,000 rpm in a microcentrifuge for 30 min at 4°C. Thirty micrograms of protein were loaded on a SDS-polyacrylamide gel, transferred to a nylon membrane, blocked for 1 h in PBS containing 0.1% Tween 20 and 5% nonfat milk, and incubated for 1 h with a rabbit polyclonal anti-rat CRHR1 antibody (1:500 dilution) in PBS containing 0.1% Tween 20 and 1% nonfat milk. The antibody was prepared at the Max Planck Institute (Gottingen, Germany), as described previously (Sydow et al., 1997). CRHR1 protein bands bound to the antibody were detected using donkey anti-rabbit γ-globulin coupled to horseradish peroxidase and the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions.

For measurement of luciferase protein by immunoblot, LVIP2.0zc cells cultured in 6-well plates were transfected with either p5UTR-luc or p5UTRm-luc. Forty-eight hours after transfection, cells were washed in PBS and lysed in 500 μl of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40 for 30 min on ice. Twenty micrograms of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, blocked in PBS containing 0.1% Tween 20 and 5% fat free milk, and incubated with a rabbit anti-luciferase polyclonal antibody (Contex Biochem, Inc., San Leandro, CA) at a 1:500 dilution for 1 h. Visualization of the luciferase bands was performed using chemiluminescence kit reagents as described for CRHR1.

For determination of the fusion upstream-GFP protein, LVIP2.0zc cells cultured in 6-well plates were transfected with either p5UTRgfp-F or p5UTRmgfp-F. Cells transfected with pcDNA3.1(+) and pEGFP-N3 were also used as negative and positive controls. Forty-eight hours after transfection, cells were lysed and subjected to Western blot analysis using a mouse anti-GFP polyclonal antibody (CLONTECH) at a 1:500 dilution, followed by incubation with goat anti-mouse lgG (1:2000) conjugated with alkaline phosphatase.

In Vitro Translation. One microgram of *Xba*I-digested pcDNA3.1(+), p5UTR-luc, p5UTRgfp-F, or p5UTRmgfp-F were transcribed from the T7 promoter and translated in a 50-μl volume containing 2 μl of [^{35}S]methionine (1,000 μCi/mmol; Amersham) and

wheat germ extract (TNT T7-coupled wheat-germ extract system; Promega). Transcription and translation reactions were incubated for 1 h at 30°C and 5 μl of the reaction mixture added to 20 μl of SDS sample buffer, and heated for 5 min at 95°C. Ten microliters of the denatured sample were separated by electrophoresis in a 10% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film overnight.

To determine whether the putative upstream peptide has any effect on translation of 5′-UTR-luc, in vitro translation reactions were performed in the presence and in the absence of the synthetic peptide (custom synthesis by Covance, Vienna, VA) or the nonspecific peptides, angiotensin I and gonadotropin-releasing hormone (Peninsula Labs, San Carlos, CA). The sequence of the uORF peptide and its scrambled sequence were MSERSRHPGR and GMRSPRERHS.

Statistical Analysis. Data are presented as the mean ± S.E.M. of the values in the number of experiments indicated under *Results* and in the figure legends. Statistical significance of the differences between experimental groups was assessed using the Student *t* test or by analysis of variance as indicated in the text of figure legends.

Results

Effect of Upstream ATG Mutation on Functional Protein Expression. The influence of the upstream ATG in the 5′-UTR of the CRHR1 mRNA on protein synthesis was first studied by examining the effect of the ATG mutation on luciferase activity after transfection of the constructs p5UTR-luc and p5UTRm-luc into COS-7 or AtT-20 cells. As shown in Fig. 2A, mutation of the upstream ATG caused marked 2.1 ± 0.29-fold and 2.4 ± 0.46-fold increases in luciferase activity in COS-7 and AtT-20 cells, respectively, compared with cells transfected with the unaltered 5′-UTR construct, p5UTR-luc ($p < 0.01$, $n = 5$).

To determine the influence of the upstream ATG on the expression of functional CRH receptors, binding of ^{125}I-Tyr-CRH to membranes and CRH-stimulated cAMP production was studied in LV1P2.0zc cells transfected with unmodified or mutant CRHR1 cDNA (p5UTR-CRHR or p5UTRm-CRHR, respectively). A radioligand binding assay showed a significant 2.4-fold increase in the number of CRH binding sites in membranes from cells transfected with the mutant upstream ATG (1254 ± 388 fmol/mg of protein), compared with membranes from cells transfected with the native CRHR1 cDNA sequence (565 ± 49 fmol/mg of protein). As shown by the parallel Scatchard plots in Fig. 3A, the binding affinity was similar in membranes from cells transfected with p5UTRm-CRHR1 or p5UTR-CRHR1 (K_d, 4.3 ± 0.7 nM versus 4.1 ± 1.6 nM, respectively, $n = 3$).

The effect of the upstream ATG mutation increasing CRH binding in LV1P2.0zc cells was also reflected in an increased ability of CRH to stimulate β-galactosidase activity, which reflects cAMP production, in this cell line expressing the β-galactosidase reporter gene driven by a cAMP-sensitive promoter. Incubation of cells with CRH for 6 and 18 h after transfection with the CRHR1 constructs, resulted in a dose-dependent increase in βγ-galactosidase activity. As shown in Fig. 3B, there was a significant increase in maximal βγ-galactosidase activity (134 ± 9%, $p < 0.05$, $n = 5$), without change in the half-maximal effective CRH concentration (EC_{50}) in cells transfected with the upstream ATG mutant receptor compared with cells transfected with the native CRHR1 cDNA. The mean EC_{50} values for the five experiments are 0.31 ± 0.08 nM and 0.32 ± 0.07 nM, for the mutant and unaltered CRHR1 constructs, respectively.

Effect of Upstream ATG Mutation on Luciferase and CRHR1 Protein Content. To further determine whether the increases in luciferase and CRH receptor activity in cells

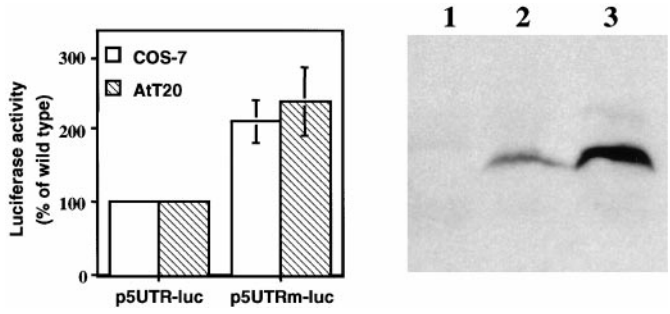

Fig. 2. Luciferase activity (A) after transfection of COS-7 and AtT20 cells with constructs containing wild-type (p5UTR-luc) or mutant (p5UTRm-luc) CRHR1 5′-UTR and the luciferase reporter gene. Luciferase activity was measured 24 h after transfection. Bars represent the mean and S.E. of the results of five experiments. B, Western blot using a luciferase antibody of LVIP2.0zc cells transfected with empty pcDNA 3.1(+) vector (lane 1), p5UTR-luc (lane 2), and p5UTRm-luc (lane 3).

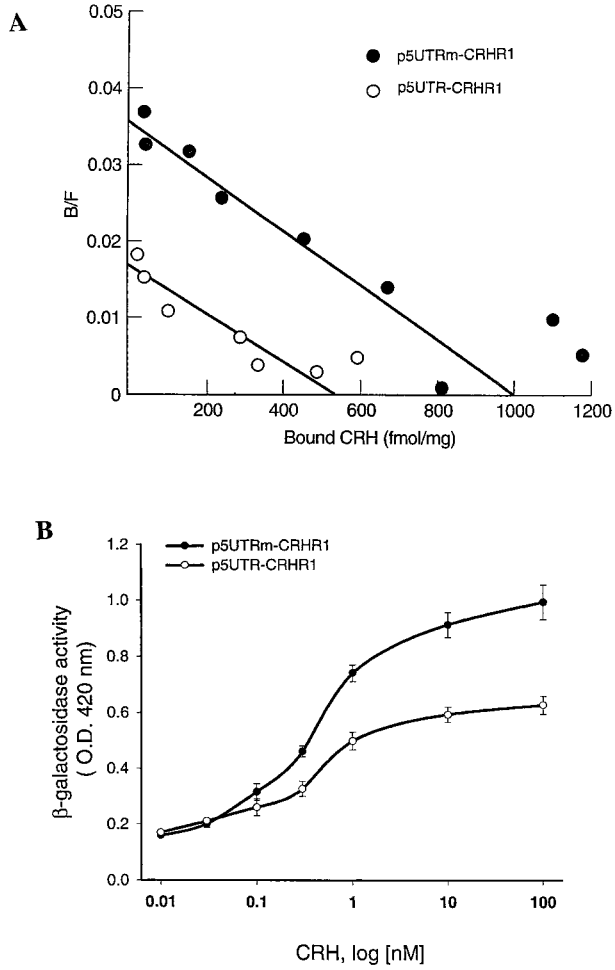

Fig. 3. Scatchard analysis of the binding of ^{125}I-Tyr-oCRH to 30,000*g* membrane fractions of LVIP2.0zc cells transfected 48 h earlier with constructs containing wild-type (p5UTR-CRHR1) or mutant (p5UTRm-CRHR1) CRHR1 5′-UTR and the main ORF of the CRH receptor. Figure is representative of three experiments. B, β-galactosidase activity measured 6 h after incubation with CRH in reporter LVIP2.0zc cells transfected 18 h earlier with p5UTR-CRHR1 or p5UTRm-CRHR1. Data points are the mean of triplicate incubations in one of five experiments.

transfected with the mutant upstream ATG was the result of increased protein synthesis, luciferase, and CRHR protein content in the cells was quantified by Western blot. As shown in Fig. 2B, immunoblot analysis of solubilized proteins from LVIP2.0zc cells transfected with the luciferase constructs, p5UTR-luc, or p5UTRm-luc, revealed a 61-kDa luciferase band, of size similar to that of recombinant luciferase used as control. Consistent with the luciferase activity experiments, the intensity of the band was higher in the cells transfected with the mutant 5′-UTR construct p5UTRm-luc than in cells transfected with the native construct p5UTR-luc. The band was not detectable from the cell transfected with the empty vector, pcDNA3.1(+).

Using an affinity-purified specific CRHR1 antibody, immunoblot analysis of solubilized membranes from cells transfected with the native CRHR1 cDNA revealed major bands of 70, 72, and 76 kDa, which are in the range of the molecular size expected for the CRHR1 (Aguilera et al, 1990; DeSouza and Grigoriadis, 1990; Sydow et al., 1997). These bands were not apparent in cells transfected with the vector alone. In three separate transfections, the intensity of the CRHR1 specific bands was higher with the construct containing the mutant upstream ATG compared with the unaltered sequence (Fig. 4, A and B).

To rule out the possibility that the changes in protein synthesis after mutation of the upstream ATG were caused by changes in transcription, CRHR1 mRNA levels were measured by RNase protection assay in LVIP2.0zc cells transfected with native or mutant CRHR1 cDNA. RNA from cells transfected with the CRHR1 constructs displayed a clear band of ~600 kb, as expected from the size of the probe. As shown in Fig. 5, no difference in CRHR1 mRNA content was observed in cells transfected with p5UTR-CRHR or p5UTRm-CRHR, indicating that mutation of the upstream ATG has no effect on transcription ($p < 0.84$; $n = 4$). On the other hand, no protected band was observed in nontransfected cells or in cells transfected with vector alone.

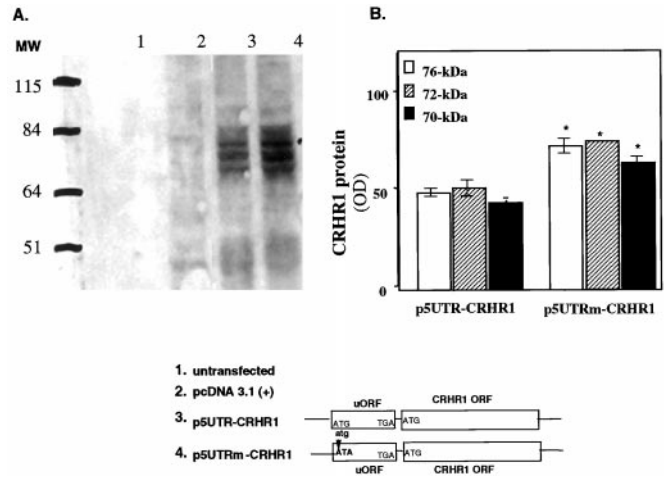

Fig. 4. Western blot analysis of CRHR1 protein after transfection of LVIP2.0zc with p5UTR-CRHR1 or p5UTRm-CRHR1. Lanes 1 and 2 in A show the lack of CRHR1 stained bands in untransfected cells or cells transfected with empty pcDNA3.1 vector. Transfection with p5UTR-CRHR1 or p5UTRm-CRHR1 reveals three protein bands labeled with the CRHR1 antibody. The pooled semiquantitation data from three experiments in B shows the intensity of all CRHR1 bands is significantly higher in cells transfected with the construct with the mutant 5′-UTR.

Translation of the Upstream ORF. To determine whether synthesis of the putative peptide is involved in the mechanism by which the upstream ORF inhibits translation of the main ORF, the ability of the upstream ATG to initiate translation was studied using the uORF-GFP fusion constructs p5UTRgfp-F and p5UTRmgfp-F. As shown in Fig. 6-A, lines 3 and 4, in vitro translation of p5UTRgfp-F resulted in a major band of about 28 kDa labeled with [^{35}S]methionine corresponding to the molecular size of the fusion protein, whereas no product was obtained with the fusion construct, p5UTRmgfp-F (Fig-6A, lanes 1 and 2).

Consistent with the in vitro translation, Western blot analysis of extracts from cells transfected with p5UTRgfp-F revealed a 28-kDa band corresponding to the size of the fusion protein (Fig. 6B). The positive control, pEGFP-N3 (CLONTECH) yielded the expected 27-kDa band corresponding to native GFP. The construct containing the ATG mutation, p5UTRmgfp-F, yielded a faint band of molecular size similar to the control GFP. This band could be caused by minor initiation of translation at an unidentified alternative codon, as reported by Kozak (1996).

Effect of Upstream Peptide on in Vitro Translation. The experiments above show that the uORF is potentially translated. To determine whether the putative peptide has any effect on the translation of the main ORF, the effect of the synthetic peptide was tested in an in vitro translation system using p5UTR-luc. In vitro translation of p5UTR-luc yielded two bands of 65 and 61 kDa (Fig. 7), whereas the control luciferase construct provided by the kit, showed a single 61-kDa band (not shown). As shown in Fig. 7A, addition of 1 to 100 μM the upstream peptide caused a dose-dependent inhibition of [^{35}S]methionine-labeled luciferase, whereas the highest concentration (100 μM) of the unrelated decapeptides angiotensin I and gonadotropin-releasing hormone were without effect. However, the scrambled sequence of the upstream peptide inhibited the in vitro translation of

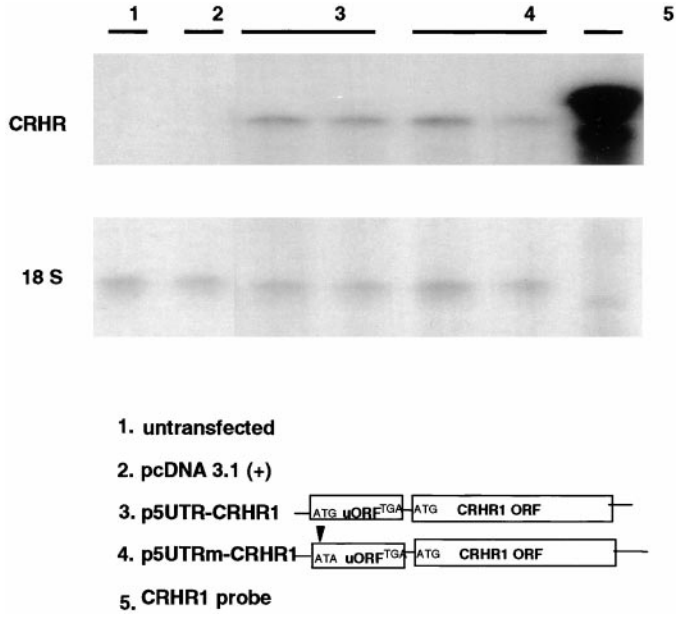

Fig. 5. CRHR1 mRNA measured by RNase protection assay in LVIP2.0zc cells transfected with p5UTR-CRHR1 or p5UTRm-CRHR1. A, ~600 bp CRHR1 protected band is evident only in cells transfected with CRHR1 constructs. Ribosomal 18 S RNA was used to correct for gel loading.

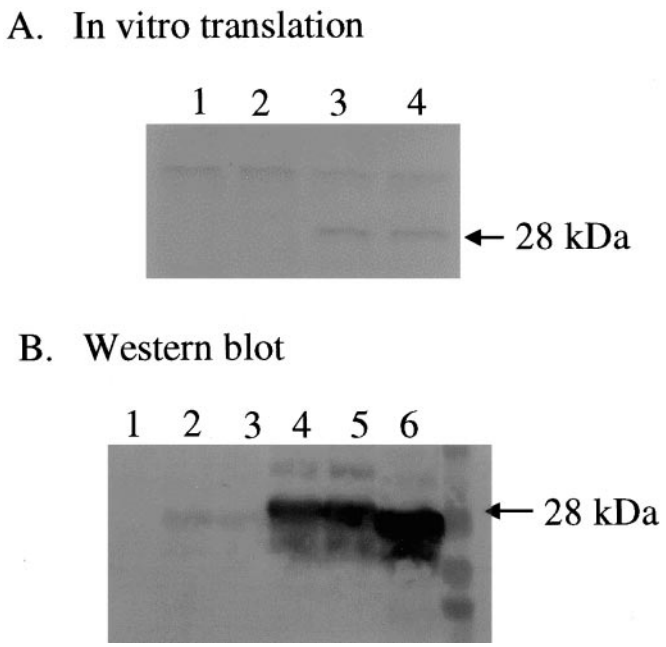

Fig. 6. In vitro translation (A) of the fusion constructs p5UTRgfp-F (lanes 3 and 4) or the ATG mutant, p5UTRmgfp-F (lanes 1 and 2). B, Western blot analysis of GFP protein (B) in LVIP2.0zc cells transfected with pEGFP-N3 as positive control (lane 6) or the fusion constructs of the uORF of CRHR1 and GFP (p5UTRgfp-F, lanes 4and 5; p5UTRmgfp-F, lanes 2 and 3). Lane 1 corresponds to cells transfected with the empty vector.

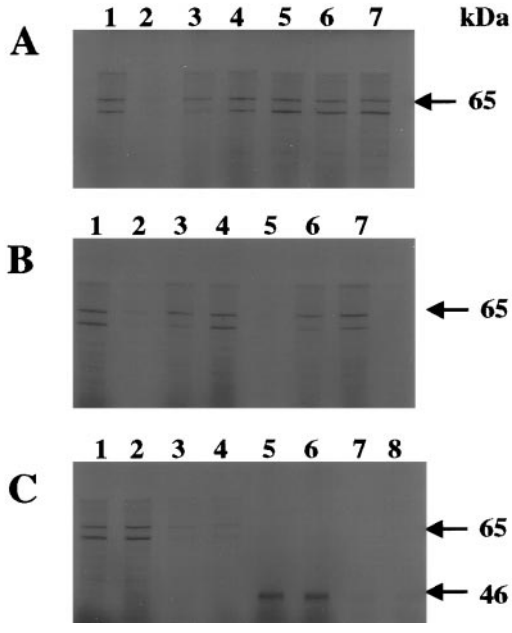

Fig. 7. Inhibition of in vitro mRNA translation by synthetic CRHR1 upstream peptide in p5UTR-luc (A and B) or a construct containing the V1b vasopressin receptor (C). A, luciferase in vitro translation with no additions (lane 1), addition of 100, 10, and 1 μM upstream peptide (lanes 2, 3, and 4, respectively), or the unrelated decapeptides angiotensin I (10 and 100 μM, lane 5 and 6) and gonadotropin releasing hormone (100 μM, lane 7). B, in vitro translation of p5UTR-luc in control conditions (lane 1) and the effects of the upstream peptide (lanes 2 to 4), or the same concentrations (100, 10, and 1 μM) of the scrambled sequence (lanes 5, 6, and 7, respectively). C, in vitro translation of luciferase without (lanes 1 and 2) and with addition (lanes 3 and 4) of 100 μM upstream peptide, and translation of V1b receptor without (lanes 5 and 6) and with 100 μM peptide (lanes 7 and 8).

luciferase with equal potency as the native sequence (Fig. 7B). Also, the upstream peptide (100 μM) inhibited the translation of vasopressin V1b receptor (Fig. 7C).

Effect of Upstream ATG Mutation on mRNA Conformation. To determine whether mutation of the upstream ATG influences the secondary structure of the mRNA, single-stranded radiolabeled RNA transcribed in vitro from p5UTR and p5UTRm was analyzed by gel electrophoresis under non-denaturing conditions. As shown in Fig. 8, RNA produced from the native 5′-UTR of CRHR1 migrated as a single band. A similar mobility was observed for RNA from the mutant 5′-UTR, but in contrast with the native sequence, the band corresponding to mRNA transcribed from the mutant 5′-UTR appeared as a doublet.

Discussion

Previous in vivo studies showing a lack of correlation between CRH binding and CRHR-1 mRNA levels have suggested that receptor levels in the pituitary may be regulated at the translational level (Rabadan-Diehl et al., 1996, 1997). Modulation of translation rate is determined not only by the stability of the mRNA, but also by structural features of the mRNA, such as a secondary structure or initiation codons in the 5′-UTR (Kozak, 1991; Geballe and Morris, 1994). Because it has been shown that the presence of ORFs in the 5′-UTR of the mRNA can influence the translation efficiency of the main ORF, this study was conducted to determine whether the 5′-UTR could be involved in the regulation of the CRHR-1 receptor translation. The present experiments provide evidence that an upstream ORF, potentially encoding 10 amino acids, in the 5′-UTR of the CRHR1, decreases translation of the CRHR or a reporter gene. Mutation of the upstream ATG in constructs with the main ORF of CRHR1 or luciferase resulted in elevation in luciferase or CRHR1 protein in transfected cells, as well as increased protein translation from these constructs in vitro. These increases in protein levels in the Western blot and in vitro translation assay occurred in the absence of any change in CRHR mRNA levels (shown by the RNase protection assay), indicating that mutation of the uORF leads to improved translation efficiency of the mRNA without changes in transcription.

The position and sequence of the uORF in the CRHR1 are well conserved in human, rat, and mouse receptor mRNA. A computer search of the GenBank database for nucleotide sequences related to uORFs failed to reveal homology with

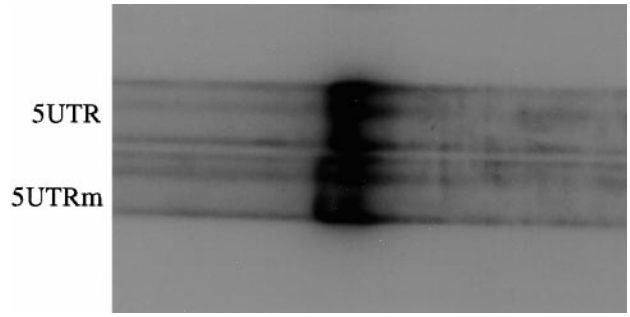

Fig. 8. Polyacrylamide analysis of in vitro-transcribed RNA for the native CRHR1 5′-UTR (5UTR) or the 5′-UTR containing an ATG-to-ATA mutation of the upstream ORF (5UTRm). The migration pattern of native and modified mRNAs showed only minor differences indicating that they have a similar conformation.

other leader peptides suggesting that it is a structure unique to CRH receptor transcripts. The fact that the 5′-UTR of the CRHR1 can regulate translation of an open reading frame other than CRHR1 (e.g., luciferase) indicates that the inhibitory effect depends on structural features of the 5′-UTR itself without interacting with distal sequences of the CRHR1 mRNA. Because polymorphism analysis of mutant and native CRHR1 mRNA 5′UTR showed minor differences in migration pattern, it is possible that conformational changes of the mRNA are responsible for the increase in translation efficiency.

The upstream ORFs could affect translation of the main ORF of the mRNA through several mechanisms including interaction of the upstream AUG with the translation preinitiation complex, or translation into an active polypeptide (Geballe and Morris, 1994; Parola and Kobilka, 1994; Reynolds et al., 1996; Hinnebush, 1997). Translation of most mRNAs involves binding of a 43 S preinitiation complex near the capped 5′ end, followed by scanning downstream until the first AUG codon is reached. This induces recruitment of the 60 S subunit resulting in formation of an 80 S complex followed by initiation of translation (Kozak, 1987; Kozak, 1991). Thus, the presence of upstream AUGs could restrict the progression of scanning ribosomes through the 5′-UTR to the start codon of the main ORF and inhibit translation without producing a functional polypeptide product (Hunt, 1985). Such a mechanism has been described in yeast in which the presence of four ORFs in the 5′-UTR of the mRNA represses translation of the transcription factor, GCN4, by interacting with the translation initiation complex and reducing its association with the AUG of the main ORF (Hinnebush, 1997).

In other hormone receptor systems such as retinoic acid, AT1 angiotensin II, and β-adrenergic receptors, there is evidence that upstream ORFs could be translated into potentially functional peptides (Geballe and Morris, 1994; Parola and Kobilka, 1994; Mori et al., 1996; Reynolds et al., 1996). In contrast to the main ORF of the CRHR1, computer analysis of the sequence reveals that the uORF is in a poor context for translation (Kozak, 1987). As reported for other mRNAs containing short upstream ORFs (Parola and Kobilka, 1994), in this study it was not possible to detect the peptide in the in vitro translation assay (not shown). However, the present demonstration that the fusion construct of the upstream ORF with luciferase is translated in vitro and in transfected cells, indicates that the upstream AUG can initiate translation, hence the uORF has the potential of being translated.

Attempts to determine whether the putative peptide has an effect on the translation of the main ORF yielded inconclusive results. Although the experiments clearly show that the peptide is inhibitory in the vitro translation assay, the concentrations required (10 and 100 μM) are unlikely to be reached in the cell, considering the low abundance of CRHR1 mRNA and the fact that such a small peptide would be rapidly degraded. However, it is not possible to rule out that peptide produced locally could reach concentrations sufficient to inhibit translation. Unexpectedly, the scrambled peptide inhibited in vitro translation as effectively as the native sequence, and the peptides also inhibited translation of a nonrelated mRNA, the V1b receptor. Although this suggests that the observed effects of the peptides are nonspecific, it should be noted that the CRHR1 upstream decapeptide peptide (as well as other reported putative upstream peptides) contains a large proportion of charged amino acids. In the CRHR1 upstream peptide, eight of the 10 amino acids are polar and five of them charged, whereas the nonrelated peptides tested (angiotensin I and gonadotropin-releasing hormone) with no inhibitory effect contained only four or five polar amino acids, of which three are charged. Because high polarity and charge will facilitate binding to RNA, it is conceivable that the upstream peptide as well as the scrambled sequence could inhibit translation by binding to RNA, regardless of the amino acid order. However, the fact that the peptide inhibited translation of a nonrelated mRNA (V1b receptor) suggests that specificity for a particular mRNA would require functional compartmentalization (for example, that the nascent peptide would immediately bind to the mRNA strand of origin). Similar problems with the interpretation of the findings are evident in other reports showing inhibition of translation of hormone receptor mRNAs with micromolar concentrations of synthetic peptides, or some cross-specificity of the effects (Parola and Kobilka, 1994; Mori et al., 1996). In general, the present results show that the uORF has the potential of being translated into a peptide capable to inhibit translation, but there is no compelling evidence suggesting that the peptide mediates the regulatory effects of the 5′-UTR on CRHR1 translation.

The role of the uORF on the physiological regulation of the CRHR-1 translation in vivo remains unknown. As other plasma membrane receptors, CRHR1 are low-abundance proteins, and receptor number undergoes rapid regulatory changes during functional alterations of the pituitary corticotroph (Aguilera, 1994). In most alterations of HPA axis activity, CRHR1 down-regulation occurs despite elevated or normal CRHR1 mRNA levels (Aguilera, 1998). Thus, regulation of translation would be an efficient mechanism to rapidly regulate receptor protein levels according to the physiological requirement. It is conceivable that access of the preinitiation complex to the upstream AUG is regulated differentially depending on the prevailing physiological conditions, thus modulating the translation efficiency of the main ORF. This could occur through structural modifications of the 5′-UTR by different promoter utilization, alternative splicing, or RNA binding proteins (Klausner et al., 1993; Kozak, 1996; Krishnamurthi et al., 1998). Concerning the CRHR1, studies in progress show that in vitro transcribed mRNA from the CRHR1 5′-UTR can bind pituitary cytosolic proteins, the extent of which changes during adrenalectomy and glucocorticoid administration (Wu et al., 2000).

In conclusion, this study demonstrates that the upstream AUG triplet present in the 5′-UTR of the CRHR1 mRNA has an inhibitory effect on translation of the CRH receptor, and that the mechanism of inhibition could involve translation of the peptide encoded by the minicistron. The findings provide a potential mechanism by which synthesis of receptor protein levels can be regulated in the absence of changes in mRNA levels.

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